

EXH. D

Construction and Characterization of a Fusion Protein of Single-Chain Anti-CD20 Antibody and Human β -Glucuronidase for Antibody-Directed Enzyme Prodrug Therapy

By Hidde J. Haisma, M. Fleur Sernee, Erik Hooijberg, Ruud H. Brakenhoff, Ida H. v.d. Meulen-Muileman, Herbert M. Pinedo, and Epie Boven

The CD20 antigen is an attractive target for specific treatment of B-cell lymphoma. Antibody-directed enzyme prodrug therapy (ADEPT) aims at the specific activation of a nontoxic prodrug at the tumor site by an enzyme targeted by a tumor-specific antibody such as anti-CD20. We constructed a fusion protein of the single-chain Fv anti-CD20 mouse monoclonal antibody (MoAb) 1H4 and human β -glucuronidase for the activation of the nontoxic prodrug N-[4-doxorubicin-N-carbonyl-(oxymethyl) phenyl] O- β -glucuronyl carbamate to doxorubicin at the tumor site. The cDNAs encoding the light- and heavy-chain variable regions of 1H4 were cloned, joined by a synthetic sequence encoding a 15-amino acid linker and fused to human β -glucuronidase by a synthetic sequence encoding a 6-amino acid linker. An antibody-enzyme fusion protein-producing cell line was established by transfection of the construct into human embryonic kidney 293/EBNA cells. The yield of active fusion protein was 100 ng/mL transfectoma supernatant. Antibody affinity,

antibody specificity, and enzyme activity were fully retained by the fusion protein. Immunoprecipitation and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the fusion protein has a relative molecular weight (Mw) of 100 kD under denaturing conditions. Gel filtration analysis indicated that the enzymatically active form of the fusion protein is a tetramer with an Mw of approximately 400 kD. The nontoxic prodrug N-[4-doxorubicin-N-carbonyl-(oxymethyl) phenyl] O- β -glucuronyl carbamate was hydrolyzed by the fusion protein at a hydrolysis rate similar to that of human β -glucuronidase. When the fusion protein was specifically bound to Daudi lymphoma cells, the prodrug induced similar antiproliferative effects as doxorubicin. Thus, it is feasible to construct a eukaryotic fusion protein consisting of a single-chain anti-CD20 antibody and human β -glucuronidase for future use in the activation of anticancer prodrugs in B-cell lymphoma.

© 1998 by The American Society of Hematology.

MOST PATIENTS WITH non-Hodgkin's lymphoma receive chemotherapy, with or without radiotherapy.¹ High-dose chemotherapy followed by stem cell rescue is now being evaluated for its effect on the clinical course of the disease and has shown improved event-free and overall survival.² Despite high response rates, 50% of all patients will eventually relapse. Therefore, alternative treatment approaches, such as the use of monoclonal antibodies (MoAbs), are evaluated. MoAbs directed against tumor-associated antigens are being applied for both the detection and the treatment of cancer. MoAb-directed therapy includes the chemically coupled radioisotopes, cytostatic agents, or toxins to MoAbs.

The CD20 antigen is a suitable candidate for targeted therapy in patients with non-Hodgkin's lymphoma: it is uniquely and abundantly expressed on normal and malignant B cells, but not on hematopoietic stem cells. It is a 33- to 35-kD integral membrane phosphoprotein, which plays a role in B-cell proliferation and differentiation. The CD20 antigen is not subject to modulation. MoAbs directed against CD20 have been successfully used in radioimmunotherapy trials in patients with B-cell malignancies.^{3,4} In the majority of patients resistant to chemo-

therapy, durable responses have been observed. Recently, phase I and phase II trials have been performed with a human/mouse chimeric anti-CD20, which shows antibody-dependent cell-mediated cytotoxicity. The response rate in the phase II study was 42%.⁵

A novel and promising strategy for targeted therapy is the use of MoAbs to direct enzymes to tumor tissue, which then convert relatively nontoxic prodrugs into active cytostatic agents (ADEPT, antibody-directed enzyme prodrug therapy).⁶⁻⁸ This approach overcomes the problem of heterogeneity in MoAb distribution, as the released drug can diffuse throughout the tumor and destroy tumor cells irrespective of their antigen expression. The catalytic activity of the enzymes can be very high and, therefore, a high concentration of drug can be achieved within the tumor, whereas little or no toxic effects are expected in nontarget tissues. The higher tumor concentration of the drug is expected to result in a higher response rate and might overcome drug resistance.

For ADEPT, we have previously used chemically coupled antibody and enzyme.^{9,10} A major disadvantage of such conjugates is their intrinsic heterogeneity due to the lack of specificity of the reagents used for conjugation. This necessitates additional purification steps and often results in reduced enzymatic activity or diminished antibody binding of the conjugate. As an alternative, recombinant DNA technology can be used to prepare conjugates consisting of a uniform product with predictable properties. Single chain antibodies (scFv), consisting of the variable VH and VL chains offer the advantage of being approximately threefold smaller than intact IgG and can penetrate tumor lesions more readily. In addition, the lack of an Fc portion should greatly reduce its immunogenicity.

We constructed a fusion protein consisting of the genes encoding the variable regions of MoAb 1H4, an MoAb reactive with the CD20 antigen^{11,12} and human β -glucuronidase. The fusion protein was expressed in eukaryotic cells, because human β -glucuronidase needs to be glycosylated to form an

From the Departments of Medical Oncology and Otolaryngology/Head and Neck Surgery, Academic Hospital Vrije Universiteit, Amsterdam; and the Department of Immunology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Huis, Amsterdam, The Netherlands.

Submitted October 30, 1997; accepted January 27, 1998.

Address reprint requests to Hidde J. Haisma, PhD, Department of Medical Oncology, Academic Hospital Vrije Universiteit, PO Box 7057, 1007 MB Amsterdam, The Netherlands; e-mail: hj.haisma.oncol@med.vu.nl.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1998 by The American Society of Hematology.

0006-4971/98/9201-0017\$3.00/0

enzymatically active tetramer.¹³ The antibody binding specificity and enzymatic activity of the fusion protein was analyzed and compared with that of 1H4 and human β -glucuronidase, respectively. Because treatment regimens for non-Hodgkin's lymphoma frequently include doxorubicin, we investigated the activating capacity of the fusion protein in the presence of a doxorubicin-glucuronide prodrug, N-[4-doxorubicin-N-carbonyl(-oxymethyl) phenyl] O- β -glucuronyl carbamate (Fig 1).¹⁴

MATERIALS AND METHODS

Reagents. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and GIBCO-BRL (Breda, The Netherlands). T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, Rnase H, Terminal Deoxynucleotidyl Transferase and DNA polymerase I (Klenow fragment) were from GIBCO-BRL. Pwo DNA polymerase (Boehringer, Almere, NL) was used for polymerase chain reaction (PCR) amplifications. All enzymes were used as recommended by the suppliers.

Oligonucleotides were synthesized by Pharmacia Biotech (Roosendaal, NL). Purification of DNA was done using spin columns (Qiagen, Hilden, Germany).

Cloning of variable regions. cDNA clones encoding the variable region domains were obtained as described.¹⁵ Briefly, RNA was isolated from the anti-CD20 hybridoma 1H4 and cDNA was synthesized by reverse transcription using an oligo-dT primer. The RNA template was destroyed with RNase H and an oligo-dC anchor sequence was added to the 3' end of the cDNA using Terminal Deoxynucleotidyl Transferase and deoxycytidine triphosphate (dCTP). This material was then used for

a PCR using primers specific for the constant regions of the heavy chain: HC1 *tgaattcggggccagtggatagac* or light chain: HL2 *tgaagcttagatgatacattgtgt*¹⁶ and an oligo-dG primer: *gtgaattcggggggggggggg*. The PCR products were cloned in pUC 19 and sequenced by the dideoxy method using a T7 kit (Pharmacia).

Construction of pscFv-CD20-human β -glucuronidase. Oligonucleotide primers were designed, encoding the linkers between the light chain and heavy chain, and the heavy chain and human β -glucuronidase. The oligonucleotide primer pair CD20VHfor 5' *gggaagcttcaccatg ggatggagtgtgtatc* 3' CD20VHback 5' *ccacacgaattcggatccgctctcctctgaggagactgtgagagtg* 3' amplified a fragment coding for the eukaryotic ribosome binding site, translation initiation codon (bold) and the N-terminal of the signal sequence of MoAb 1H4 and part of a synthetic 15-amino acid linker (gly₄-ser)₂ as described by Huston.¹⁷ The primers contained an *Hind*III site at the 5' end and a *Bam*HI and *Eco*RI site at the 3' end indicated in italics.

The oligonucleotide pair CD20VLfor 5' *tgtgaagcttggatccggcggaggcggaagcgggtggcggaggctctcaaatgttctctccagtc* 3' CD20VLback 5' *ccaagaattctcgtagtc tccggaaccttggattccagcttggtgc* 3' amplified a fragment encoding part of the linker between VH and VL and the MoAb 1H4 light chain without its signal sequence. The primers contained an *Hind*III and *Bam*HI site at the 5' end and an *Xho*I and *Eco*RI site at the 3' end (italics). The 3' terminus also contained a *Bsp*EI site followed by a stop codon (bold) to enable expression of scFv-CD20 without fusion to human β -glucuronidase. The *Bsp*EI site is preceded by part of a gly₄-ser₂ linker for the joining to β -glucuronidase. The oligonucleotide pair GUSfor 5' *tgtgaagcttccggagggttctgctgcaggcggcgatgctgtacc* 3' GUSback 5' *ccaagaattctcgtagtcagtagcagcttcatgccaactctttattcc* 3' was used to amplify a DNA fragment encoding part of the gly₄-ser₂ linker and human β -glucuronidase¹⁸ without its signal peptide. The PCR primers contained an *Hind*III and *Bsp*EI site at the 5' end and an *Xho*I and *Eco*RI site at the 3' end.

All PCR fragments were cloned into pUC19 after digestion with *Hind*III and *Eco*RI and the sequences were checked by the dideoxy method using T7 DNA polymerase (Pharmacia). pscFv-CD20 was constructed by inserting the *Hind*III/*Bam*HI fragment of partially digested VL CD20 into the VH CD20 vector digested with the same enzymes. β -Glucuronidase cDNA was amplified with the indicated primers, cut with *Hind*III and *Kpn*I, and cloned into a *Kpn*I and *Hind*III-digested pUC19 vector. The *Kpn*I-*Eco*RI fragment of the original cDNA encoding the enzyme was then ligated into this vector, cut with *Kpn*I and *Eco*RI, thus reconstructing the open reading frame encoding β -glucuronidase. The pscFv-CD20- β -glucuronidase was constructed by insertion of the *Bsp*EI/*Eco*RI fragment encoding β -glucuronidase into the pscFv-CD20 (Fig 2).

Expression and purification of scFv-CD20- β -glucuronidase. The insert containing scFv-CD20- β -glucuronidase was prepared by *Eco*RI digestion, blunt ends preparation by treatment with dNTPs (2'-deoxynucleoside 5'-triphosphates) and Klenow polymerase, followed by subsequent *Hind*III digestion. The isolated *Hind*III-*Eco*RI/blunt fragment of scFv-CD20- β -glucuronidase was ligated into *Xba*I (blunt)/*Hind*III digested vector pcDNA3 (Invitrogen, Leek, NL). An enzyme control vector was constructed using the human β -glucuronidase-encoding cDNA.¹⁸ The plasmids were transfected into COS-7 cells by diethyl aminoethyl (DEAE)-dextran for transient expression.¹⁹ Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS) for 72 hours and the medium containing scFv-CD20- β -glucuronidase or β -glucuronidase was collected. To obtain stable transfectants, the fusion protein insert was isolated from the pcDNA3 vector by digestion with *Hind*III and *Xho*I and ligated into the pCEP4 vector (Invitrogen) digested with the same enzymes. The pCEP4 vector is an Epstein-Barr virus (EBV)-based vector, which is maintained and replicated extrachromosomally in primate and canine cells. This vector was used to transform 293/EBNA (Invitrogen) cells using DOSPER (Boehringer). Transfected cells were

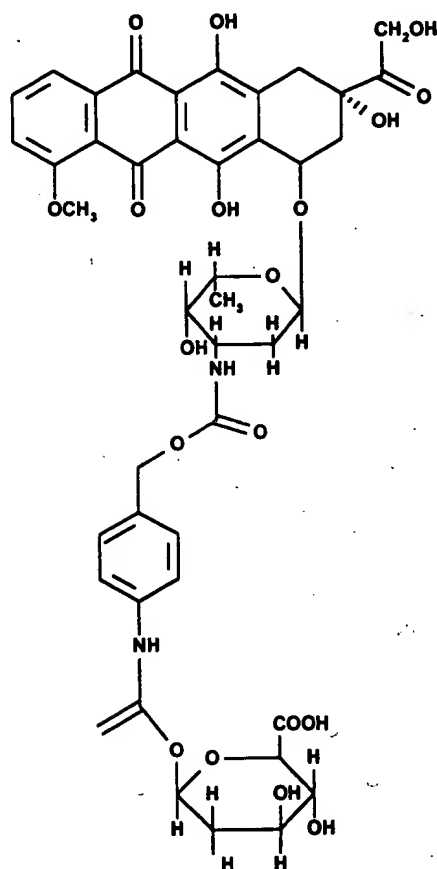


Fig 1. Chemical structure of prodrug N-[4-doxorubicin-N-carbonyl(-oxymethyl) phenyl] O- β -glucuronyl carbamate (DOX-GA3).

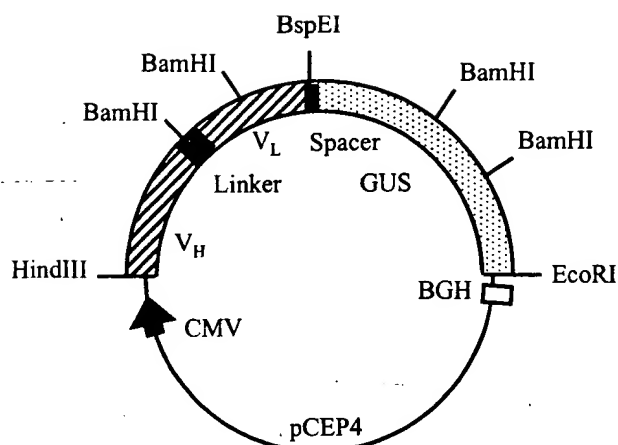


Fig 2. Schematic representation of the pCEP4 scFv-CD20- β -glucuronidase expression vector. Transcription is driven by the human cytomegalovirus (CMV) enhancer-promotor sequence. The polyadenylation signal and transcription termination signal are from the bovine growth hormone gene.

selected in medium containing 400 μ g/mL hygromycin B (Boehringer). After 2 to 3 weeks resistant clones were isolated and recloned by limiting dilution. The scFv-CD20- β -glucuronidase fusion protein was purified from transfectoma supernatants by ion-exchange chromatography as described for human β -glucuronidase.²⁰

Characterization. The molecular weight of the fusion protein or β -glucuronidase was determined by immunoprecipitation and gel filtration. For immunoprecipitation, COS-7 cells were metabolically labeled 48 hours after transfection. First the cells were starved for 1 hour in DMEM plus 5% dialyzed fetal bovine serum, which lacked methionine and cysteine. For labeling, the same medium was used containing 100 μ Ci of Trans³⁵S-label (ICN, Zoetermeer, The Netherlands) for 4 hours. Cell extracts and supernatants were then immunoprecipitated with rabbit antihuman β -glucuronidase as described¹⁸ and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli.²¹

Gel filtration was performed with an SX200 column (1.6 \times 60 cm; Pharmacia, Roosendaal, NL) in phosphate-buffered saline (PBS) to estimate the molecular mass of the native fusion protein. The column was run at 0.5 mL/min and fractions (0.5 mL) were assayed for β -glucuronidase activity. The column was calibrated with bovine serum albumin (67 kD), IgG (150 kD), and β -galactosidase (480 kD).

β -Glucuronidase activity was measured fluorometrically. To 50 μ L of transfection supernatant or purified enzyme 100 μ L substrate (4-methylumbelliferyl- β -glucuronide, 1 mmol/L in 100 mmol/L sodium acetate buffer pH 4.5) was added and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 1 mL 0.1 mol/L Glycine-NaOH pH 10.5. Fluorescence was measured using an excitation wavelength of 370 nm and an emission wavelength of 450 nm.

The binding of the fusion protein was determined with Daudi cells.²² Purified fusion protein was added to Daudi cells (10^5) at various concentrations for 1 hour at 4°C in 100 μ L. Cells were washed three times with PBS and resuspended in 100 μ L 4-methylumbelliferyl- β -glucuronide in buffer. After 2 hours at 37°C, 1 mL 0.1 mol/L glycine-NaOH was added and the fluorescence was measured as described above. Binding was determined by subtracting the values obtained with cells preincubated with an excess of anti-CD20 MoAb BCA-B20⁶ from the fluorescence of the samples.

Prodrug activation. The kinetics of the hydrolysis by human β -glucuronidase or fusion protein was determined with the prodrug N-[4-doxorubicin-N-carbonyl-(oxymethyl) phenyl] O- β -glucuronide¹⁴ at a concentration of 100 μ mol/L in PBS containing 0.1% BSA at pH 6.8 and at 37°C. Hydrolysis was followed by high-performance

liquid chromatography (HPLC) analysis using a silica-C18 column (4.6 \times 100 mm, 3 μ m CP; MicroSpher, Chrompack, Middelburg, NL) and an isocratic eluent, which consisted of 2 mmol/L triethylamine in 20 mmol/L NaH₂PO₄ (pH 4.0)-acetonitrile (2:1, vol/vol) at a flow rate of 1 mL/min. Before each analysis, samples were diluted in ice-cold methanol to precipitate proteins. The eluate was analyzed with a fluorescence detector using an excitation wavelength of 480 nm and an emission wavelength of 580 nm. Peak areas were used to calculate the concentrations of drug and prodrug.

In vitro antiproliferative effects. Daudi lymphoma cells²² were incubated with fusion protein (10 μ g/mL) or PBS for 1 hour at 4°C, washed three times and seeded into 96-well plates at 2×10^4 cells/well. To determine the specificity of the fusion protein, a separate set of Daudi cells was pretreated (30 minutes, 4°C) with an excess of anti-CD20 antibody BCA-B20,⁶ washed, and then incubated with fusion protein. The cells were then exposed to the prodrug N-[4-doxorubicin-N-carbonyl-(oxymethyl) phenyl] O- β -glucuronide at concentrations ranging from 0.01 to 50 μ mol/L. In separate wells an excess of β -glucuronidase was present to determine the antiproliferative effects of prodrug completely hydrolyzed by the enzyme. After 24 hours, 200- μ L medium was added and the cells were allowed to proliferate for another 72 hours. Cells were fixed with trichloroacetic acid and stained with Sulforhodamine B as described.¹² The antiproliferative effects were determined and expressed as IC50 values, the concentrations that give 50% growth inhibition compared with control growth.

RESULTS

Cloning of the 1H4 variable cDNAs and construction of scFv-CD20- β -glucuronidase fusion protein. The cDNAs encoding the 1H4 variable domains were cloned by reverse transcriptase PCR from total RNA isolated from the 1H4 hybridoma. The nucleotide sequence of the heavy and light variable chain cDNA was determined. At least three independent clones were sequenced to ensure that the DNA polymerase used in the PCR induced no mutations. The nucleotide and deduced amino acid sequence of the cDNA encoding the scFv-CD20 is shown in Fig 3. The sequences were used to design primers for the construction of the fusion protein.

The plasmid pCD20- β -glucuronidase was constructed as shown in Fig 2. A 15-amino acid linker containing (gly₄ser)₃, as described previously, separated the variable VH and VL antibody domains.¹⁷ The cDNA encoding human β -glucuronidase¹⁸ was inserted in frame of the scFv separated by a 6-amino acid linker segment. The signal peptides of VL and human β -glucuronidase were removed. The open reading frame encoded a recombinant protein, which after cleavage of the secretory signal sequence and C-terminal processing, was composed of 878 amino acids with a predicted molecular weight (Mw) of 100 kD.

Expression and characterization of fusion protein. The fusion protein was expressed in eukaryotic cells because human β -glucuronidase needs to be glycosylated to form an enzymatically active tetramer.¹³ The supernatants of the transfected COS-7 cells contained approximately 10 ng/mL of enzymatically active fusion protein, which was approximately 10-fold less than that of COS-7 cells transfected with vector encoding human β -glucuronidase. For continuous production of fusion protein, 293/EBNA cells were transfected with pCEP4 vector encoding the fusion protein. Clones were selected with hygromycin B and subcloned by limiting dilution. Culture superna-

1H4 Vh

leader peptide
 Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
 ATG GGA TGG AGT TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA

1981
 Gly Val His Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu
 GGT GTC CAC TCC CAG GTG CAA CTG CAG CAG CTT GGG GCT GAG CTG

Val Lys Pro Gly Ala Ser Val Lys Met Ser Cys Lys Thr Ser Gly
 GTG AAG CTT GGG GCT TCA GTG AAG ATG TCC TGC AAG ACT TCT GGC

1982
 Tyr Thr Phe Thr Ser Tyr Asn Val His Trp Val Lys Gln Thr Pro
 TAC ACA TTT ACC AGT TAC AAT GTG CAC TGG GTA AAG CAG ACA CTT

1983
 Gly Gln Gly Leu Glu Trp Ile Gly Ala Ile Tyr Pro Gly Asn Gly
 GGA CAG GGC CTG GAA TGG ATT GGA GCT ATT TAT TAT CCA GGA AAT GGC

1984
 Asp Thr Ser Phe Asn Gln Lys Phe Lys Gly Ala Thr Leu Thr
 GAT ACT TCC TTC AAT CAG AAG TTC AAA GGC AAG GCC ACA TTG ACT

Ala Asp Lys Ser Ser Ser Thr Val Tyr Met Gln Leu Ser Ser Leu
 GCA GAC AAA TCC TCC AGC ACA GTT TAC ATG CAA CTC AGC AGC CTG

1985
 Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg Ser Asn Tyr
 ACA TCT GAG GAC TCT GCG GTC TAT TAC TGT GCA AGA TGG AAT TAC

1986
 Tyr Gly Ser Ser Tyr Val Trp Phe Phe Asp Val Trp Gly Ala Gly
 TAC GGT AGT AGC TAC GTT TGG TTC GAT GTC TGG GGC CCA GGG

Thr Thr Val Thr Val Ser Ser Ala Lys Thr Thr Pro Pro Ser Val
 ACC AAG GTC ACC GTC TCC TCA GGC AAA AAG ACA GGC CCA TCT GGC

Tyr Pro Leu Ala Pro Asn
 TAT CCA CTG GGC GGG AAT ATC

Codon Vh NCI primer

1H4 Vh

leader peptide
 Met Asp Phe Gln Val Gln Ile Phe Ser Phe Leu Leu Ile Ser Ala
 ATG GAT TTT CAA GTG CAG ATT TTC AGC TTC CTG GTA ATC AGT GCT

1981
 Ser Val Ile Met Ser Arg Gly Gln Ile Val Leu Ser Gln Ser Pro
 TCA GTC ATA ATG TCC AGA GGA CAA ATT GTT CTC TCC CAG TCT CCA

Thr Ile Leu Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys
 ACA ATC CTG TCT GCA TCT CCA GGG GAG AAG GTC ACA ATG ACT TCC

1982
 Arg Ala Ser Ser Ser Val Ser Tyr Met Asp Trp Tyr Gln Gln Lys
 AAG GGC AAG TCA AGT GTC AGT TAC ATG GAC TGG TAC CAG CAG AAG

1983
 Pro Gly Ser Ser Pro Lys Pro Trp Ile Tyr Ala Thr Ser Asn Leu
 CCA GGA TCC TCC CCC AAA CCC TGG ATT TAT GGC ACA TCC AAC CTG

1984
 Ala Ser Gly Val Pro Ala Arg Phe Ser Gly Ser Gly Thr
 GCT TCT GGA GTC GCT GCT CAC TTT AGT GGC AGT GGG TCT GGG ACC

1985
 Ser Tyr Ser Lys Thr Ile Ser Arg Val Glu Ala Glu Asp Ala
 TCT TAC TCT CTC ACA ATC AGC AGA GTG GAG GCT GAG GAT GCT GGC

1986
 Thr Tyr Tyr Cys Gln Gln Trp Ile Ser Asn Pro Pro Thr Phe Gly
 ACT TAT TAC TCC CAG CAG TGG ATT AGT AAC CCA GGC AAG TTC GGT

Ala Gly Thr Lys Leu Glu Leu Lys Arg Ala Asp Ala Pro Thr
 GCT GGG AAG CAG CTG GAG CTG AAA GGC GGT GAT GCT GCA GCA AGC

Val Ser Ile ...
 GGA TCC ATC TAA GCT T

Codon Vh NCI primer

Fig 3. Nucleotide and deduced amino acid sequence of the VH and VL of anti-CD20 MoAb 1H4. The primers used for cloning are underlined. The cleavage site of the leader peptide (bold) is indicated by the arrow.

tants from these cells produced approximately 100 ng/mL fusion protein.

Fusion protein or β -glucuronidase was analyzed by immunoprecipitation followed by SDS-PAGE. The fusion protein migrated with an apparent Mw of 100 kD, whereas β -glucuronidase monomers showed an Mw of approximately 80 kD (Fig 4). Gel filtration analysis of the fusion protein showed that it migrates as a tetrameric protein with the expected Mw of approximately 400 kD (data not shown).

The binding of the fusion protein to Daudi lymphoma cells is shown in Fig 5. The specificity of binding was determined with the blocking anti-CD20 antibody BCA-B20. Binding of the fusion protein was less than 5% in the presence of excess

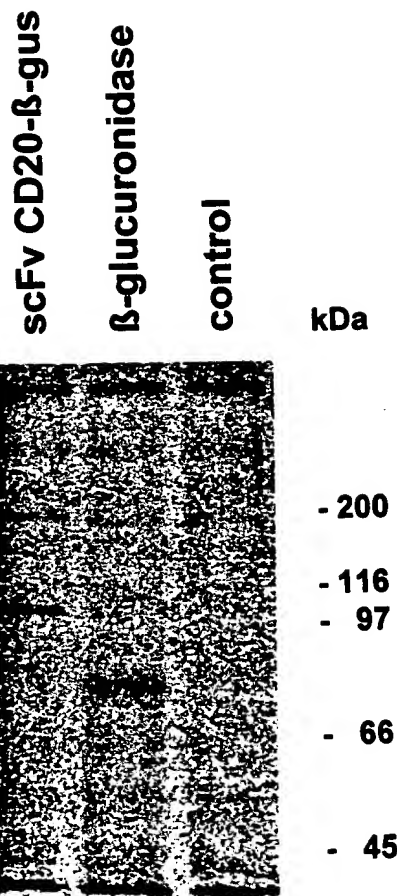


Fig 4. Immunoprecipitation of scFv-CD20- β -glucuronidase. COS-7 cells were transfected with scFvCD20- β -glucuronidase or β -glucuronidase cDNA. The cells were labeled 48 hours after transfection with 35 S-methionine/cysteine for 4 hours, the supernatant was immunoprecipitated with rabbit anti- β -glucuronidase antibody and analyzed by SDS-PAGE.

antibody. From the binding data, the apparent affinity of the fusion protein was calculated to be 3×10^9 L/mol.

Prodrug activation and antiproliferative effects. The enzymatic activity of the fusion protein was compared with that of native recombinant β -glucuronidase using the prodrug N-[4-

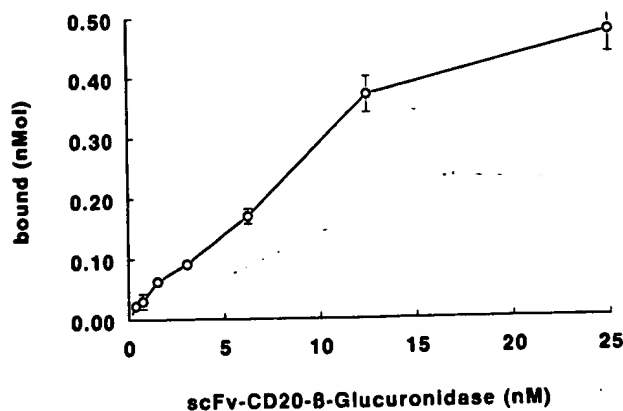


Fig 5. Binding of scFv-CD20- β -glucuronidase to Daudi lymphoma cells. Cells were incubated with fusion protein. Cells were washed and the binding was determined by measuring the β -glucuronidase activity.

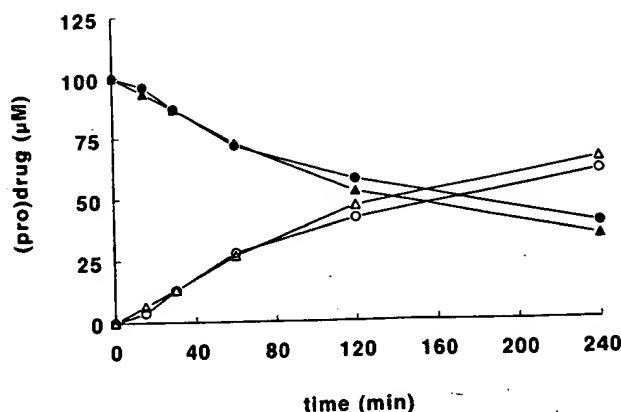


Fig 6. Hydrolysis of the prodrug N-[4-doxorubicin-N-carbonyl (-oxymethyl) phenyl] O-β-glucuronyl carbamate (100 μmol/L, closed symbols) to the cytotoxic drug doxorubicin (open symbols) by human β-glucuronidase (Δ and ▲, 1 μg/mL) or scFv-CD20-β-glucuronidase (○ and ●, 1 μg/mL) as determined by reversed-phase HPLC and fluorescence detection.

doxorubicin-N-carbonyl(-oxymethyl) phenyl] O-β-glucuronyl carbamate as a substrate. At 100 μmol/L prodrug concentration, the activation rates for the conversion of prodrug to drug were similar ($T_{1/2} = 130$ minutes) for both the fusion protein and the enzyme (Fig 6), showing that the fusion protein possessed the full enzymatic activity of human β-glucuronidase.

Daudi lymphoma cells were used to determine the specific activation of the prodrug N-[4-doxorubicin-N-carbonyl (-oxymethyl) phenyl] O-β-glucuronyl carbamate by scFv-CD20-β-glucuronidase fusion protein. In the presence of an excess of β-glucuronidase, the prodrug was activated to doxorubicin. Similarly, preincubation of Daudi cells with the fusion protein increased the antiproliferative effects of the prodrug as a result from activation (Fig 7). Specificity was evident, because cells preincubated with an excess of BCA-B20 blocking antibody and fusion protein showed no increase in growth inhibition when compared with incubation with the prodrug alone.

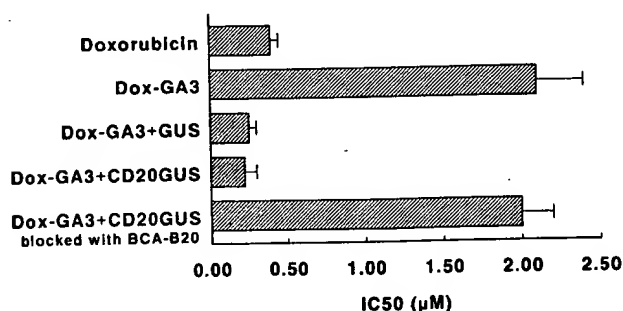


Fig 7. Antiproliferative effects of doxorubicin, prodrug N-[4-doxorubicin-N-carbonyl(-oxymethyl) phenyl] O-β-glucuronyl carbamate (DOX-GA3), and prodrug combined with scFv-CD20-β-glucuronidase. Cells were incubated with fusion protein, blocking antibody, and fusion protein, or PBS. Cells were then seeded in 96-wells plates and exposed to drug or prodrug for 24 hours. After 72 hours, cell growth was measured with a protein dye, Sulforhodamine B.

DISCUSSION

We have constructed an expression plasmid for the production of a single-chain antibody-enzyme fusion protein composed of the anti-CD20 antibody 1H4 and the human lysosomal enzyme β-glucuronidase. The scFv-CD20-β-glucuronidase fusion protein was expressed in eukaryotic cells and was shown to exist in a tetrameric form. The fusion protein bound to CD20-expressing Daudi lymphoma cells; the antigen specificity was confirmed by competition experiments. The enzymatic activation of the prodrug N-[4-daunorubicin-N-carbonyl (-oxymethyl) phenyl] O-β-glucuronyl carbamate by the fusion protein was at the same rate as that for β-glucuronidase. The fusion protein, when specifically bound to Daudi lymphoma cells, completely activated the nontoxic prodrug and growth inhibition was similar to that obtained with doxorubicin.

Several chemically prepared β-glucuronidase immunoconjugates have been described for activation of anticancer prodrugs. We have used both *Escherichia coli* (*E coli*)-derived β-glucuronidase and human β-glucuronidase conjugated to MoAb 323/A3 for the activation of anthracycline prodrugs such as epirubicin-glucuronide and daunorubicin-glucuronide.^{11,12,14} Roffler et al²³ have used *E coli* β-glucuronidase for the activation of an aniline mustard prodrug. Antibody-enzyme conjugates prepared with chemical cross-linkers have the disadvantage that the conjugates differ in the amount and position of the cross-links resulting in inconsistent fractions of enzyme and antibody activity. Recombinant DNA techniques provide the means of directly producing a fusion protein with defined characteristics. Furthermore, when scFv fragments are used, the antibody size is reduced from 150 kD to approximately 25 kD. The smaller size of a fusion protein prepared with scFv fragments instead of IgG will result in better tumor penetration properties.²⁴ In addition, scFv are expected to be less immunogenic than whole IgG antibodies.^{25,26}

The yield of enzymatically active fusion protein was low: 10 ng/mL in COS-7 transfectoma supernatants. This was approximately 10-fold lower than the expression level of β-glucuronidase in the same vector. Southern blot analysis of transcripts indicated that RNA levels were reduced in cells transfected with the fusion protein when compared with those of the enzyme (data not shown). We could increase the expression level 10-fold in 293/EBNA cells, which were transfected with the pCEP4 vector. This vector is EBV-based and is maintained and replicated extrachromosomally in primate and canine cells.

Several antibody-enzyme fusion proteins have been described. Goshorn et al²⁷ and Rodrigues et al²⁸ have developed Fv-β-lactamase fusions. These fusion proteins, prepared with a bacterial enzyme, showed selective activation in vitro of cephalosporin prodrugs of a nitrogen mustard and doxorubicin, respectively. Bosslet et al^{29,30} have developed an antibody-human β-glucuronidase fusion protein. This fusion protein was tested in mice bearing human tumor xenografts and found to be rapidly cleared from the blood resulting in tumor:blood ratios of >100:1 at 7 days after injection. The combination of this fusion protein with a doxorubicin prodrug showed superior growth inhibition when compared with doxorubicin alone. The fusion protein of Bosslet et al²⁹ differs from our scFv-CD20-β-glucuronidase fusion protein in that the enzyme was fused to a humanized VH containing an IgG3 hinge region, which was

joined to the VL of the antibody by disulfide bond formation. The antibody moiety was a F(ab)₂ fragment and not a scFv. The apparent native Mw of this fusion protein was 250 kD, which is conflicting with the observation that β -glucuronidase needs a tetrameric configuration with an apparent Mw of 400 kD to be enzymatically active.¹³ Apparently, processing of the c-terminal end of β -glucuronidase in the baby hamster kidney cells used by Bosslet et al differs from that of other cells, which leads to dimeric enzyme molecules.³¹

The observed Mw of 400 kD of the scFv-CD20- β -glucuronidase fusion protein is similar to the size of the chemically linked conjugate with Mw 400 kD.²⁰ Unfortunately, the large size of the tetrameric protein will impede tumor penetration. We have constructed a similar fusion protein between human β -galactosidase, which is a monomeric lysosomal enzyme, and scFv-CD20. The scFv-CD20- β -galactosidase fusion protein had an apparent Mw of 100 kD, both on SDS-PAGE, as well as by gel filtration (data not shown) and should have improved in vivo tumor penetration properties.

Of importance, the design of the expression cassette used for scFv-CD20- β -glucuronidase enables the rapid exchange of both the scFv and the enzyme. Thus, fusion proteins may be constructed that target different tumor types and use prodrugs activated by other enzymes.

ACKNOWLEDGMENT

We gratefully acknowledge the gift of the cDNA encoding human β -glucuronidase and the rabbit antiserum against human β -glucuronidase from Prof. W. Sly.

REFERENCES

1. Armitage JO: Treatment of non-Hodgkin's lymphoma. *N Engl J Med* 328:1023, 1993
2. Philip T, Guglielmi C, Hagenbeek A, Somers R, Vanderlelie H, Bron D, Sonneveld P, Gisselbrecht C, Cahn JY, Harousseau JL, Coiffier B, Biron P, Mandelli F, Chauvin F: Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma. *N Engl J Med* 333:1540, 1995
3. Kaminski MS, Zasadny KR, Francis IR, Milik AW, Ross CW, Milik AW, Estes J, Tuck M, Regan D, Fisher S, Glenn SD, Wahl RL: I-131-anti-B1 radioimmunotherapy for B-cell lymphoma. *J Clin Oncol* 14:1974, 1996
4. Press OW, Eary JF, Appelbaum FR, Martin PJ, Nelp WB, Glenn S, Fisher DR, Porter B, Matthews DC, Gooley T, Bernstein ID: Phase II trial of 131-I-B1 (anti-CD20) antibody therapy with autologous stem cell transplantation for relapsed B cell lymphomas. *Lancet* 346:336, 1995
5. Maloney DG, Liles TM, Czerwinski DK, Waldichuk C, Rosenberg J, Grillo Lopez A, Levy R: Phase I clinical trial using escalating single-dose infusion of chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in patients with recurrent B-cell lymphoma. *Blood* 84:2457, 1994
6. Bagshawe KD: Antibody-directed enzyme prodrug therapy. *Drug Dev Res* 34:220, 1995
7. Knox RJ, Connors TA: Antibody-directed enzyme prodrug therapy. *Clin Immunother* 3:136, 1995
8. Senter PD, Wallace PM, Svensson HP, Vruthula VM, Kerr DE, Helstrom I, Helstrom KE: Generation of cytotoxic agents by targeted enzymes. *Bioconjug Chem* 4:3, 1993
9. Haisma HJ, Boven E, Van Muijen M, De Jong J, Van der Vijgh WJF, Pinedo HM: A monoclonal antibody-beta-glucuronidase conjugate as activator of the prodrug epirubicin-glucuronide for specific treatment of cancer. *Br J Cancer* 66:474, 1992
10. Haisma HJ, Van Muijen M, Pinedo HM, Boven E: Comparison of two anthracycline-based prodrugs for activation by a monoclonal antibody-beta-glucuronidase conjugate in the specific treatment of cancer. *Cell Biophys* 24/25:185, 1994
11. Hooijberg E, Sein JJ, Van den Berk PCM, Hekman A: Characterization of a series of isotype switch variants of a new CD20 monoclonal antibody. *Hybridoma* 15:23, 1995
12. Hooijberg E, Sein JJ, Van den Berk PCM, Hart AAM, Van der Valk MA, Kast WM, Melief CJM, Hekman A: Eradication of large human B cell tumors in nude mice with unconjugated CD20 monoclonal antibodies and interleukin 2. *Cancer Res* 55:2627, 1995
13. Shipley JM, Grubb JH, Sly WS: The role of glycosylation and phosphorylation in the expression of active human beta-glucuronidase. *J Biol Chem* 268:12193, 1993
14. Houba PHJ, Leenders RGG, Boven E, Scheeren JW, Pinedo HM, Haisma HJ: Characterization of novel anthracycline prodrugs activated by human β -glucuronidase. *Biochem Pharmacol* 52:455, 1996
15. Frohman MA, Dush MK, Martin GR: Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci USA* 85:8998, 1988
16. Caton AJ, Brownlee GG, Staudt LM, Gerhard W: Structural and functional implications of a restricted antibody response to a defined antigenic region on the influenza virus hemagglutinin. *EMBO J* 5:1577, 1986
17. Huston JS, Levinson D, Mudgett-Hunter M, Tai MS, Novotny J, Margolies MN, Ridge RJ, Brucoleri RE, Haber E, Crea R: Protein engineering of antibody binding sites, recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc Natl Acad Sci USA* 85:5879, 1988
18. Oshima A, Kyle JW, Miller RD, Hoffman JW, Powell PP, Grubb JH, Sly WS, Tropak M, Guise KS, Gravel RA: Cloning, sequencing and expression of cDNA for human beta-glucuronidase. *Proc Natl Acad Sci USA* 84:685, 1987
19. Brakenhoff RH, Knippels EM, van Dongen GAMS: Optimization and simplification of expression cloning in eukaryotic vector/host systems. *Anal Biochem* 218:460, 1994
20. Houba PHJ, Boven E, Haisma HJ: Improved characteristics of a human β -glucuronidase antibody conjugate after deglycosylation for use in antibody-directed enzyme prodrug therapy. *Bioconjug Chem* 7:606, 1996
21. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680, 1970
22. Klein E, Klein G, Nadkarni JS, Nadkarni JJ, Wigzell H, Clifford P: Surface IgM-kappa specificity on a Burkitt lymphoma cell in vivo and in derived culture lines. *Cancer Res* 28:1300, 1968
23. Roffler SR, Wang SM, Chern JW, Yeh MY, Tung E: Antineoplastic glucuronide prodrug treatment of human tumor cells targeted with a monoclonal antibody-enzyme conjugate. *Biochem Pharmacol* 42:2062, 1991
24. Jain RK: Haemodynamic and transport barriers to the treatment of solid tumours. *Int J Radiat Biol* 60:85, 1991
25. LoBuglio AF, Wheeler RH, Trang J, Haynes A, Rogers K, Harvey EB, Sun L, Ghayeb J, Khazaeli MB: Mouse/human chimeric monoclonal antibody in man, kinetics and immune response. *Proc Natl Acad Sci USA* 86:4220, 1989
26. Meredith RF, LoBuglio AF, Plott WE, Orr RA, Brezovich IA, Russell CD, Harvey EB, Yester MV, Wagner AJ, Spencer SA: Pharmacokinetics, immune response, and biodistribution of iodine-131-labeled chimeric mouse/human IgG1,k 17-1A monoclonal antibody. *J Nucl Med* 32:1162, 1991
27. Goshorn SC, Svensson HP, Kerr DE, Somerville JE, Senter PD, Fell HP: Genetic construction, expression, and characterization of a

single chain anti-carcinoma antibody fused to beta-lactamase. *Cancer Res* 53:2123, 1993

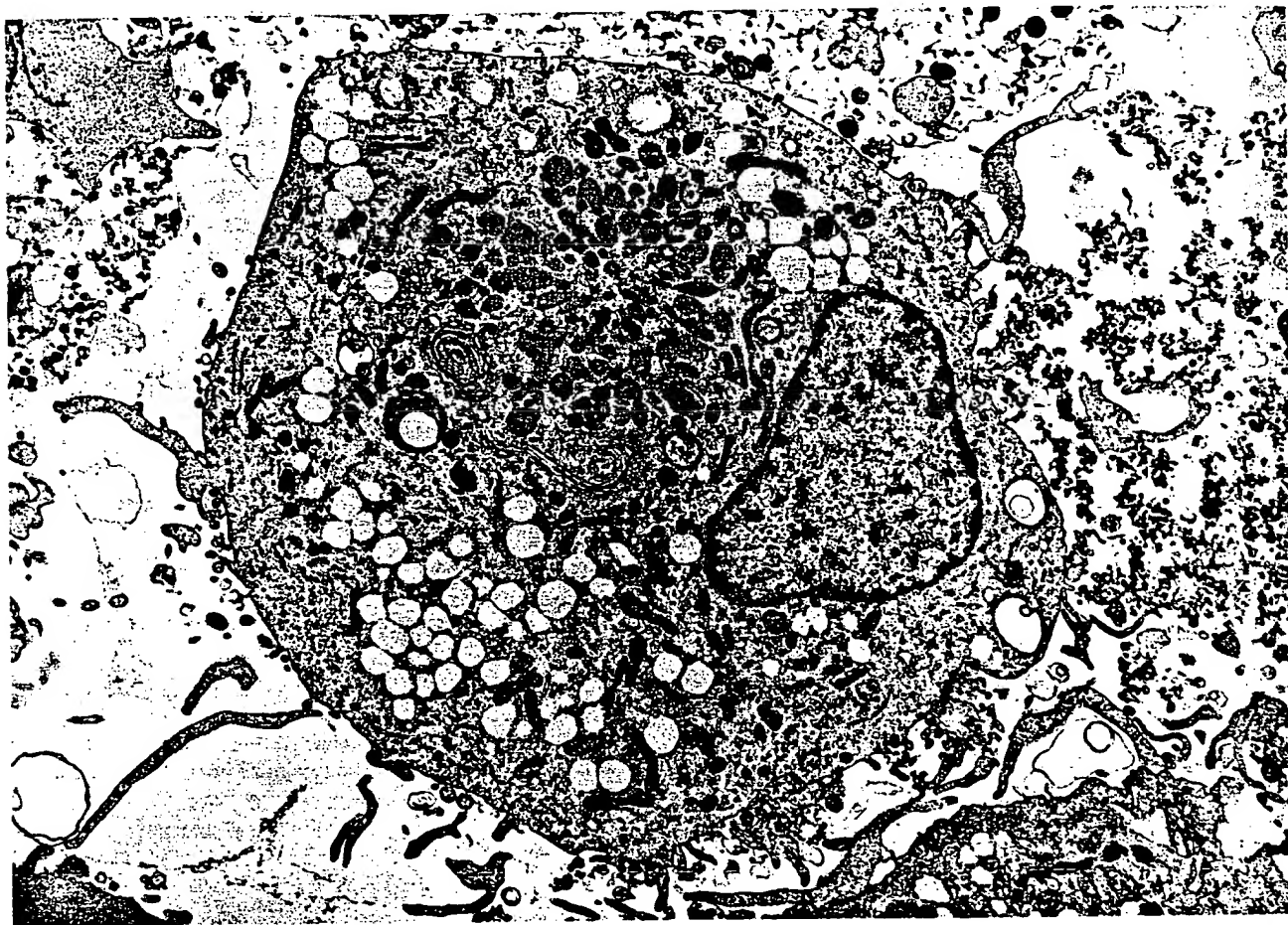
28. Rodrigues ML, Presta LG, Kotts CE, Wirth C, Mordenti J, Osaka G, Wong WL, Nuijens A, Blackburn B, Carter P: Development of a humanized disulfide-stabilized anti-p185HER2 Fv-beta-lactamase fusion protein for activation of a cephalosporin doxorubicin prodrug. *Cancer Res* 55:63, 1995

29. Bosslet K, Czech J, Lorenz P, Sedlacek HH, Schuermann M,

Seeman G: Molecular and functional characterization of a fusion protein suited for tumour-specific prodrug activation. *Br J Cancer* 65:234, 1992

30. Bosslet K, Czech J, Hoffman D: Tumor-selective prodrug activation by fusion protein-mediated catalysis. *Cancer Res* 54:2151, 1994

31. Gehrmann MC, Oppen M, Sedlacek HH, Bosslet K, Czech J: Biochemical properties of recombinant human β -glucuronidase synthesized in baby hamster kidney cells. *Biochem J* 301:821, 1994



Human dendritic cell derived from peripheral blood progenitors with recombinant interleukin-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The cells have long microvillous surface processes and numerous cytoplasmic lysosomes in addition to a well-formed golgi apparatus (uranyl acetate, lead citrate, original magnification $\times 2,500$). (Courtesy of Jonathan W. Said, Kai Chien, Walter Verbeek, Jeffrey Weber, H. Phillip Koeffler, UCLA Center for the Health Sciences, University of Southern California, and Cedars Sinai Medical Center, Los Angeles, CA.)

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to Commissioner of Patents and Trademarks, Washington, D.C. 20231, on

Date: May 25, 1998 Atty's Reg. #19,805

John Q. McQuillan
JOHN Q. McQUILLAN